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Neutrophil Extracellular Traps (NETs) profiles in patients with incident SLE and lupus nephritis

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Running headline: NETs in SLE/Lupus Nephritis

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ABSTRACT 250

Objective. Neutrophil Extracellular Traps (NETs) expose modified antigens for auto-antibodies in vasculitis (SVV). Little is known on levels and removal pathways of NETs in Systemic Lupus Erythematosus (SLE), especially in lupus nephritis (LN). We determined circulating levels and defined NETs removal in large subsets of incident SLE patients, a part with newly onset nephritis.

Methods. Serum levels of NETs (ELISA), DNase1/DNase1L3 (ELISAs) and DNase activity (functional assay) were determined in 216 incident SLE patients, 103 had incident LN, in 50 patients with other primary glomerulonephritis and in healthy controls. *Ex vivo* NETs production by neutrophils purified from a random selection of patients was quantified as elastase/DNA release and by immunofluorescence techniques.

Results. Serum NETs were very high in iSLE/iLN compared to all groups of controls and correlated with anti-dsDNA, C3-C4 and proteinuria; incident LN had the highest levels. DNase activity was decreased in iLN compared to SLE (20% had one half DNase activity) despite similar serum levels of DNase1/DNase1L3. In these cases, pre-treatment of serum with Protein A restored DNase efficiency; one patient was homozygous for a c.289_290delAC variant of *DNASE1L3. Ex vivo* NETs production by neutrophils purified from LN, SLE and normal controls was similar in all cases.

Conclusions. iLN patients have increased circulating NETs and reduced DNase activity, the later being explained by the presence of inhibitory substances in circulation and/or by rare DNase1L3 mutations. Accumulation of NETs derives from a multi-factorial mechanism, is associated and may contribute to disease severity in SLE, in particular to renal lesions.

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INTRODUCTION 302

The release of Neutrophil Extracellular Traps, or NETosis, is one of the first defense line utilized by neutrophils against bacteria, virus, protozoa and other pathogens (1-3). It starts with the de-condensation and release of nuclear chromatin outside the cell and leads to the formation of a physical net where pathogens are entrapped and killed by elastase, defensin and reactive oxygen species (ROS) (1, 4, 5).

Despite their beneficial effects in host defense, NETs occur at the expense of potential injury to the host. The formation and removal of NETs should be timely regulated and failure to do so may lead to unfavorable consequences. NETosis may be, in particular, implicated in the pathogenesis of autoimmune conditions since DNA and post-translational modified proteins in the NETs may become antigenic (6-11). This view is also supported by the observation that pathogens, which activate NETosis, frequently function as trigger or enhancing factor for autoimmune diseases (12).

In early studies, patients with systemic lupus erythematosus (SLE) were shown to display higher NET levels than healthy controls, possibly due to a defective NET degradation (13). Within SLE patients, NETs levels and NET-degrading ability have been associated with disease severity (14) where, in small series of patients, higher NETs levels correlate with the development of lupus nephritis (LN). Nonetheless the importance of the topics, only few studies have focused on the kinetics of NETs in SLE and data on levels of circulating NETs in LN are still scanty. Our study fills up these gaps: taking advantage of the large cohort of patients with SLE and lupus nephritis (LN) recruited within the Zeus project(15), we measured circulating NETs remnants, kinetics of NETs formation and removal by DNase in large cohorts of SLE and LN patients. Potential factors modifying DNase activity (ie. circulating inhibitors, mutations of *DNASE*s) were, in parallel, analyzed.

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PATIENTS AND METHODS 1344

Study design. Samples from SLE patients were obtained from the bio-bank of the Zeus study, a prospective, multicenter study collecting blood samples and clinical information from SLE patients (15) (NCT02403115). A written consent was obtained before sampling. The data base and samples collection is located at the Giannina Gaslini Institute of Genoa (I). Diagnosis of SLE was done according to the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus (SLICC) (16). Controls were 50 healthy donors of the hospital staff (19-50 yrs); 20 patients with IgA GN and 30 with Membranous nephropathy were also studied. All SLE were incident patients (i). Also LN patients were incident; they were recruited immediately at the onset of renal symptoms that coincided, in general, with the time of renal biopsy. Blood samples were obtained at this time (**Table 1A**). The iLN cohort included two populations: a first group presented the renal pathology as first symptom (iLN as onset), a second group presented the renal flare after 1 or more years from the SLE diagnosis (iLN in SLE) (see **Table 1B**).

In all patients, disease activity and type of organ involvement were scored according to the SLEDAI 2000-2K index (17). Lupus nephritis was defined according to WHO classification on the basis of immunofluorescence for IgG, IgA, IgM, C1q, C3 deposition and classical histology stainings (hematoxylin/eosin, Masson's trichrome, silver methenamine, and periodic–acid Schiff). Patients with severe infections, malignancies, positivity for chronic hepatitis B, HBV or Hepatitis C virus, breast-feeding or pregnant were excluded. Therapies manly consisted in steroids and hydroxylchloroquine in SLE and steroids plus cytotoxic drugs or cyclosporine in LN patients (**Table 1B**).

Permission and Registration. We obtained written approval of the protocol by the local Independent Ethics Committee (Comitato Etico Regione Liguria) on October 24th, 2014 (n

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407REG2014). The study was approved by the Italian Drug Agency (AIFA) and was registered at https://clinicaltrials.gov (study number: NCT02403115).

Serum NET remnants quantification. Levels of NET remnants were determined in serum and plasma utilizing an ELISA assay in accord to Hakkim et al. (13) with some modification (18). The assay determines the myeloperoxidase (MPO)-DNA complex and consists in blocking MPO with specific antibodies on solid phase and determining the free edge of the complex with unconjugated anti-dsDNA antibodies (3519 DNA, AbCam, Cambridge, UK). Anti-MPO monoclonal antibody (5 µg/ml) (Clone 2A11, Serotec, Bio-Rad, CA, USA) were coated overnight at 4°C in 96-well maxi-sorp-nunc-immuno plates (ThermoFisher Scientific, MA, USA) in 3% BSA in PBS. One-hundred µl of (1:50) diluted sera were added per well and incubated overnight at 4°C. After 3 washes with PBS and 0.05% v/v of tween-20 (PBS-T), samples were incubated 4 hours with anti-double-stranded DNA polyclonal antibody (Abcam, Cambridge, UK). After three washes with PBS-T, HRP anti-Human IgG were added and incubated 1 hour and washed again three times with PBS before adding the peroxidase substrate (TMB, Bio-Rad). Absorbance at 450 nm was measured using Mark microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, Ca).

DNase activity and Protein A for removing inhibitors. DNase activity was determined with a one-step assay based on the decrease of fluorescence intensity of degrading Picogreen DNA dye/double-stranded DNA (dsDNA) complex in solution. Serum and plasma (100 µl) diluted 1:50 with 100 mM Tris-HCl, 20 mM $CaCl_2$ and 2 mM $MgCl_2$ (pH 6.8) were placed in ultraviolet light-transmissive high-quality 96-well micro-plates (Brand, Wertheim, Germany). After adding high polymeric dsDNA (SIGMA-Aldrich, MI, USA) and Picogree DNA dye (ThermoFisher Scientific, MA, USA) pre-heated at 37°C, a first fluorescent measurement was performed after 5 minutes (T_0).

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Then, the plate was incubated overnight at 37°C, sealed from the top with adhesive sticker to inhibit evaporation during incubation step and protected from light until the final measurement after 16 hours (T_{16}). Measurements of Picogreen DNA dye fluorescence intensity were performed in a fluorescence reader at 520 nm (three reads with 20 µs of integration time). The percentage of decrease of fluorescence intensity was determined by subtracting the value obtained at T_0 with the value at T_{16}. Results are given as % of degraded DNA for well.

DNase activity in 'low activity samples' was also measured after Protein A (staphylococcus aureus) treatment to remove potential inhibitors of DNases. In this case, 100 µl of Protein A (Sigma Aldrich, St Louis, MO) in PBS were incubated with 100 µl of serum for 2 hours. Unbound material was recovered after centrifugation (800 g x 30 min) and re-tested for DNase activity.

DNASE1 and DNASE1L3 quantification. A home-made ELISA assay was utilized to test DNAse1 serum levels. Anti-DNAse1 rabbit polyclonal antibodies (Abcam) were coated in 96-well maxi-sorp-nunc-immuno-plates (ThermoFisher Scientific)and maintained overnight at 4°C. After blocking in 3% BSA in PBS, 100 µl of standard or 1:50 diluted sera were added to each well and incubated overnight at 4°C. Wells were washed three times with PBS and 0.05% v/v of tween-20 (PBS-T). Then, plates were incubated 4 hours with anti-DNase1 mouse polyclonal antibody (Abnova, Taipei, Taiwan). Each well was, then, washed again, added with 100 µl of substrate solution and blocked with 50 µl of stop solution; reaction was read at 450 nm as above. Results are given as ng/ml. The assay was validated by comparison with a commercial kit for DNase1 (Catalog n LS-F27682 LSBio Inc., Seattle, USA).

For DNASE1L3 we utilized a specific for DNase 1L3 ELISA (kit purchased from LSBioInc., Seattle, USA) according to the manufacturer´s instructions; details are given in the Supplementary methods. Results are given as ng/ml.

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***DNASE1L3* sequencing.** The analysis was done with Next Generation Sequencing in the Laboratory of Neurogenetics and Neuroinflammation at the Image-Institut des maladies genetiques, Paris (Prof Y. Crow) (19).

Anti-DNA antibodies. Anti-DNA antibodies determination was done with 2 methods, one was the commercial assay DNA-DIAMEDIX (Delta Biologicals, Rome, Italy) and the second was a home-made western-blot. Details are given in the Supplements Methods. The agreement between DIAMEDIX and the home-made assay was measured using the Cohen kappa (k=0.61; 0.50-0.72 CI at 95%) and the Sperman's correlation coefficients (0.67 with 0.59-0.75 CI at 95%). Sensitivity, and specificity were 90% and 70%, respectively.

***Ex vivo* NET production.** The study on *ex vivo* NET production was done in representative part of patients recruited for the main study groups above (ie. 18 with active iLN, 15 with iSLE and 27 controls). Neutrophils were isolated from heparinized peripheral blood under sterile conditions, using dextran sedimentation followed by Ficoll gradient centrifugation (20) (see supplement methods for more details). Neutrophil suspensions were allowed to adhere onto 24-well plastic dishes and were cultivated as described in supplementary methods. NET release was evaluated in two ways: one is quantitative and utilizes the elastase and DNA release in resting and Phorbol Myristate Acetate (PMA) stimulated cells; the second technique determines NETs DNA and Histone 1-3 composition after stimulation of neutrophils with serum from SLE and controls.

Elastase-DNA assay. To quantify NETs production, it was used the Cayman's NETosis assay kit (cat. No 601010, Cayman Chamical, MI, USA) according to the manufacturer´s instructions that

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deterimes the release of elastase from immobilized. Briefly, 100 µl of standard or culture supernatants per well, pre-heated to 37°C, were incubated with 100 µl of the 1:30 diluted NET assay neutrophil elastase substrate for 2 hours at 37°C before reading at 405 nm.

Serum-stimulated neutrophils. Neutrophils were re-suspended in 2% Human Serum Albumin and allowed to adhere on poly-L-lysine–coated glass slides for 40 min, incubated with 0,05 ml SLE and control sera and then fixed with 3,7% paraformaldehyde. Immunofluorescence for Histone 1 and 3 are described in Supplementary Methods.

Statistical analysis. Comparison of data sets were done using Mann-Whitney or Kruskal–Wallis tests respectively for two or more of two unpaired samples. Spearman correlations were calculated for defining any relationship among biochemical parameters and biomarkers of lupus activity. Two-tailed P-values ≤ 0.05 were considered significant.

In Receiver operating characteristic (ROC) analysis, proteins with an area under the curve (AUC) ≤ 0.5 were excluded.

Normal Limits. Normal limits for all the parameters above were calculated from ROC curves; the Cut Off represented the value that minimizes the geometric distance from 100% sensitivity and 100% specificity on the ROC curves (21, 22).

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RESULTS 805

Clinical features of the different groups of patients. The main clinical differences between the 3 sub-groups of patients (incident SLE, incident LN as onset, incident LN after SLE) are reported in **Table 1A** and **1B**. Age was comparable excepting for 3 pts under 16 years in the group with iLN as onset. With the exception of the renal involvement that was uniquely present in all iLN and joints that were comparable in iSLE and iLN, there were a few minimal disparities in other organ pathologies (**Table 1A**). With reference to renal parameters (histology, proteinuria, renal function etc.) no difference was observed in iLN with nephritis occurring as onset of the disease or after at least 1 year from the diagnosis of SLE.

Circulating NETs remnants are high in SLE and lupus nephritis. Serum NETs remnant levels were significantly higher in patients with iLN and iSLE compared to healthy controls and to patients with other primary glomerulonephritis ($p<0.0001$) (**Figure 1A, Supplement Figure 1**). NETs levels were comparable in the two sub-groups of iLN and, in both, were higher compared to SLE ($p<0.02$). ROC curves showed AUCs of 0.82 and 0.92 of the DNA-MPO assay (measuring NETs remnants) to diagnose SLE or LN, respectively (**Figure 1B**) (21, 22).

Serum NETs did not correlate with SLEDAI (**Supplement Table 1**) nor with age and therapies and in particular with steroid doses. On the contrary, NETs had a series of correlations that are shown by the correlogram in **Figure 1C** which includes, in red, positive relationships with circulating anti-dsDNA, anti-C1q antibodies (**Figure 1D**), proteinuria and, in blue, negative relationships with complement and CRP.

DNase activity is reduced in lupus nephritis. DNase activity was reduced in iLN patients compared to SLE and controls (**Figure 2A**) reaching, in some cases, levels one half the normal activity. The

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two subgroups of iLN had comparable DNase activity. Overall, DNase activity did not correlate with circulating NETs (See Heat Map in **Figure 1C**). However, the lowest levels were found in patients with high circulating NETs: 20% of LN patients with serum NETs > 0.5 (RU/ml) had DNase under the limit of normality (**Figure 2B, C**). In the same Figure 2B it is shown that SLE patients with high NETs (>0.5RU/ml) but without nephritis (and also normal controls) had normal DNase activity suggesting that low DNase is a peculiarity of patients with LN.

Sera with DNase activity in the lower range (that are indicated in squares in Figure2b) were pre-treated with Protein A to remove potential inhibitors. This pre-treatment increased DNase activity up to normal levels in 5 samples, implying that the removal of serum elements with affinity for the dye had restored DNase activity in patients with very low functional levels (**Figure 2C**).

Mutations of *DNASE1L3.* One patient (indicated with a triangle in Figure 2A) was found to carry the a c.289-290delAC homozygous variant of *DNASE1L3*. He was a boy of 10 yrs who presented a severe form of lupus nephritis that progressed very rapidly to end stage renal failure. After a few months of hemo-dialysis he received a renal transplant. Details on the clinical outcome and on therapies will be reported separately from here.

Circulating levels of DNASE1 and DNASE1L3. The serum levels of these two enzymes that are deputed to NETs removal (23, 24) were similar in LN and SLE patients and in healthy controls (**Figure 3A, 3B**). DNASE1 and DNASE1L3 levels were not correlated with serum NETs nor with DNase activity (**Supplement Figures 2A, 2B, 2C**). Actually, DNASE1L3 levels showed a broad variation both in iLN and controls: levels were almost 0 in a few cases; the carrier of the c.289-290delAC homozygous variant (indicated with an arrow in Figure 3B) had a low levels. DNASE1 and DNASE1L3 levels did not correlate (**Figure 3C**). This excludes that there is a sort of 'compensatory

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effect' between the two enzymes implying there are different mechanisms regulating their expression.

Ex vivo NETs formation. We studied *ex vivo* NETs production by neutrophils obtained from subsets of patients recruited in the main study (neutrophils from 15 iSLE, 18 iLN and 27 normals) in two ways: **1**-by determining the release of elastase and DNA from neutrophils stimulated with phorbol-12-myristate-13-acetate (PMA); **2**-by staining for Histones 1-3 by and DNA neutrophils stimulated by patient and control sera.

Resting neutrophils from patients with SLE and LN released lower levels of elastase than control cells; after stimulation with PMA, the elastase release increased significantly in the three groups, control neutrophils still presenting the major increment (**Figure 4A**). DNA and elastase released from stimulated neutrophils were highly correlated (**Figure 4B**) implying that elastase reflects the DNA-NET complex.

After stimulation with SLE and control sera, the same neutrophils produced extracellular traps containing Histone 1, Histone 3 and DNA (**Figure 4C**). Results were not quantitative and did not allow to produce a clear discrimination between different sera and cells.

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DISCUSSION 1003

Modifications of the mechanisms regulating NETosis have been associated with autoimmunity based on the concept that DNA and post-translational modified proteins in NETs may become antigenic(6-8). Failure of NETs removal has been, in particular, considered a trigger for developing renal lesions (LN) in patients with SLE (13). Lupus nephritis is an autoimmune condition that develops in about 50% of all SLE patients (25, 26) and causes end stage renal failure in a significant portion of this cohort. In this study, we investigated NETs formation/removal in SLE patients seeking to define whether kinetics of NETs is different in those patients who develop LN. For this study it was utilized a large population of patients with incident SLE/LN that included patients recruited at the time of the onset of symptoms that for incident LN corresponded to the time of renal biopsy. For the large number of patients, incident LN was also subdivided in cases with LN as first symptom or incident LN occurring in patients with an already known diagnosis of SLE.

We report here new findings that provide elements of interest on NETosis and its correlation with autoimmunity and with renal manifestations of SLE: ***1***-circulating serum NET remnants were high in lupus nephritis and SLE compared to other control groups and correlated with proteinuria; the increment was more evident in incident LN; ***2***- serum NETs correlated with anti-dsDNA, C3 and C4 but not with SLEDAI-2k; ***3***-a significant reduction of DNase activity was observed limited to LN patients, in spite serum DNASE1/DNASE1L3 levels were normal; inhibitors of DNase activity were shown in a few cases, one boy had a c.289_290delAC homozygous variant in *DNASE1L3.* Therefore, increasing NETs levels seems the result of a multi-factorial mechanism that includes increased autoimmune activity (that should reflect increased production) and failure to remove DNA.

To the best of our knowledge, this is the first study showing a marked increase of circulating NETs in a large cohort of patients with incipient SLE and in particular in patients with incipient lupus

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nephritis. The later finding is of particular interest suggesting that serum NETs are in some way involved or participate in the pathogenesis of renal lesions. On the contrary, NETs levels did not correlated with clinical signs of lupus acute activity such as the SLEDAI-2k index thus representing a parameter indicating activity in the long term.

Ex vivo studies with isolated and PMA stimulated neutrophils did not show increase production of NETs in LN and SLE. Qualitative assays with neutrophils stimulated with various sera showed extracellular expression DNA and Histones in all conditions but could not add any quantitative hint. Overall, *ex vivo* studies utilizing all neutrophils cannot be considered representative of specific subsets of activated neutrophils (27) and are not conclusive.

A main finding here presented is that DNase activity is defective in patients with LN. Digestion of circulating DNA and of DNA in NETs is mediated by DNASE1 and DNASE1L3 (28). In our patients, serum DNASE1 and DNASE1L3 levels were normal (albeit within a broad range in different cohorts) and no correlation was found between serum DNASE1 and/or DNASE 1L13 levels suggesting that the two enzymes are not substitute of each other and are regulated by separate mechanisms.. The only study available in the literature reported low DNASE1 activity serum in 23 patients with SLE who were studied in combination with patients with microscopic polyangiitis (29). No data are available relative to serum levels of DNASE1IL3. Other Authors (13, 30) have shown the existence of circulating anti-NET antibodies that inhibit DNASEs in a subset of patients with SLE, defined as non-degraders, who were prone to develop LN. Genetic data in humans carrying mutations of *DNASEs* (19, 31-33) and results deriving from molecular manipulation of *DNASEs* in mice (28, 34-36) indicate an association between mutations of *DNASEs*, reduced DNase activity and autoimmune activity, in particular, with the development of renal lesions.

We could confirm here, in a large cohort of patients with Lupus nephritis, that both mechanisms (ie. presence of inhibitors and genetic defects) modify DNase activity *in vivo* and that they are

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associated with a specific phenotype of SLE with renal lesions. Actually, DNase activity was restored by ProteinA suggesting that circulating inhibitors of DNASEs are probably IgG that have been removed by this protein; this finding confirms and strengthens the original hypothesis made by Hakkim (13) about the existence of circulating antibodies that reduce NETs removal in SLE patients prone to develop nephritis. A c.289_290delAC homozygous variant in *DNASE1L3* has been detected in one young boy (the youngest of the entire cohort) who presented a very early development of LN and rapid evolution to end stage renal failure (clinical details and therapy will be presented and discussed elsewhere). The same mutation of *DNASE1L3* has been already described in few other children with early-onset hypo-complementaemic urticarial vasculitis with glomerulonephritis (31). Overall, it seems reasonable that molecular defects should be detected in young people, whereas circulating inhibitors could occur at an older age. Considering the different clinical approaches that should be utilized in the two different conditions (ie. in presence of inhibitory substances vs. molecular molecular defects) we propose here to introduce both the determination of serum NET remnants and the functional analysis of DNase as screening tests in clinical settings; in those cases who present low DNase activity (and we suggest only in this subset of patients) it would be useful to proceed with the characterization of circulating inhibitors and with molecular sequencing of *DNASE1* and *DNASE1L3.*

In conclusion, our results show a relationship between NET levels and removal with lupus nephritis in patients with SLE. The present data on reduced DNase activity also support the idea that, in some cases and specifically in patients with nephritis, NETs accumulate in serum for a defective removal and circulating inhibitors of DNase activity are potentially responsible for this phenomenon. More rare mutations in *DNASE1L3* produce similar modifications. Altogether, these findings on NET remnants levels and their kinetics of production and removal represent a further advancement with new diagnostic and therapeutic potential implications.

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Author contribution

GMG was the PI of the study**.** He was involved in study design and coordination, patients' recruitment, data managing and supervision, manuscript writing and discussion. **MB** had a key role in lab analysis, proteomics, supervision, statistics and data managing; **AB** was involved the patient recruitment, data collection, manuscript discussion, coordination of the study; **RB, MG, GC** were involved in lab analysis; **PR** and **PB** performed STED analysis; **AP** performed the mass spectrometry analysis; **AV, PC, FP, PM, SV** were involved in some experimental work and discussed critically the manuscript. **LS, BS**, **LC, GM, FF, MF, GP, LA, RAS, GP, MB, AM, GAR, FP, FP, SN, FM, GE, GG, DS, FS, AR, AT** were involved in patient recruitment, data managing, manuscript discussion.

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Competing interests. Authors declare no conflicts of interest. A patent on the use of anti-enolase antibodies in diagnosis of LN is pending.

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REFERENCES

1. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science 2004;303:1532-5.

2. Nathan C. Neutrophils and immunity: Challenges and opportunities. Nat Rev Immunol 2006;6:173-82.

3. Papayannopoulos V, Zychlinsky A. Nets: A new strategy for using old weapons. Trends Immunol 2009;30:513-21.

4. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 2007;176:231-41.

5. Urban C, Zychlinsky A. Netting bacteria in sepsis. Nat Med 2007;13:403-4.

6. Knight JS, Carmona-Rivera C, Kaplan MJ. Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases. Front Immunol 2012;3:380.

7. Rohrbach AS, Slade DJ, Thompson PR, Mowen KA. Activation of pad4 in net formation. Front Immunol 2012;3:360.

8. Darrah E, Andrade F. Nets: The missing link between cell death and systemic autoimmune diseases? Front Immunol 2012;3:428.

9. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. Toll-like receptor 7 and tlr9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. Immunity 2006;25:417-28.

10. Wang Y, Wysocka J, Sayegh J, Lee YH, Perlin JR, Leonelli L, et al. Human pad4 regulates histone arginine methylation levels via demethylimination. Science 2004;306:279-83.

11. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. Pad4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J Exp Med 2010;207:1853-62.

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12. Christensen SR, Kashgarian M, Alexopoulou L, Flavell RA, Akira S, Shlomchik MJ. Toll-like receptor 9 controls anti-DNA autoantibody production in murine lupus. J Exp Med 2005;202:321-31.

13. Hakkim A, Furnrohr BG, Amann K, Laube B, Abed UA, Brinkmann V, et al. Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. Proc Natl Acad Sci U S A 2010;107:9813-8.

14. Leffler J, Martin M, Gullstrand B, Tyden H, Lood C, Truedsson L, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol 2012;188:3522-31.

15. Bonanni A, Vaglio A, Bruschi M, Sinico RA, Cavagna L, Moroni G, et al. Multi-antibody composition in lupus nephritis: Isotype and antigen specificity make the difference. Autoimmun Rev 2015;14:692-702.

16. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the systemic lupus international collaborating clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum 2012;64:2677-86.

17. Touma Z, Urowitz MB, Ibanez D, Gladman DD. Sledai-2k 10 days versus sledai-2k 30 days in a longitudinal evaluation. Lupus 2011;20:67-70.

18. Bruschi M, Petretto A, Bertelli R, Galetti M, Bonanni A, Pratesi F, et al. Post-translational modified proteins are biomarkers of autoimmune-processes: Netosis and the inflammatory-autoimmunity connection. Clin Chim Acta 2017;464:12-6.

19. Rice GI, Melki I, Fremond ML, Briggs TA, Rodero MP, Kitabayashi N, et al. Assessment of type i interferon signaling in pediatric inflammatory disease. J Clin Immunol 2017;37:123-32.

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20. Weiss J, Kao L, Victor M, Elsbach P. Oxygen-independent intracellular and oxygen-dependent extracellular killing of escherichia coli s15 by human polymorphonuclear leukocytes. J Clin Invest 1985;76:206-12.

21. Zweig MH. Roc plots display test accuracy, but are still limited by the study design. Clin Chem 1993;39:1345-6.

22. Zweig MH, Campbell G. Receiver-operating characteristic (roc) plots: A fundamental evaluation tool in clinical medicine. Clin Chem 1993;39:561-77.

23. Napirei M, Ludwig S, Mezrhab J, Klockl T, Mannherz HG. Murine serum nucleases--contrasting effects of plasmin and heparin on the activities of dnase1 and dnase1-like 3 (dnase1l3). FEBS J 2009;276:1059-73.

24. Lo MS. Monogenic lupus. Curr Rheumatol Rep 2016;18:71.

25. Cameron JS. Lupus nephritis. J Am Soc Nephrol 1999;10:413-24.

26. Borchers AT, Naguwa SM, Shoenfeld Y, Gershwin ME. The geoepidemiology of systemic lupus erythematosus. Autoimmun Rev 2010;9:A277-87.

27. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. J Immunol 2011;187:538-52.

28. Sisirak V, Sally B, D'Agati V, Martinez-Ortiz W, Ozcakar ZB, David J, et al. Digestion of chromatin in apoptotic cell microparticles prevents autoimmunity. Cell 2016;166:88-101.

29. Nakazawa D, Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, et al. Enhanced formation and disordered regulation of nets in myeloperoxidase-anca-associated microscopic polyangiitis. J Am Soc Nephrol 2014;25:990-7.

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30. Zykova SN, Tveita AA, Rekvig OP. Renal dnase1 enzyme activity and protein expression is selectively shut down in murine and human membranoproliferative lupus nephritis. PLoS One 2010;5.

31. Ozcakar ZB, Foster J, 2nd, Diaz-Horta O, Kasapcopur O, Fan YS, Yalcinkaya F, et al. Dnase1l3 mutations in hypocomplementemic urticarial vasculitis syndrome. Arthritis Rheum 2013;65:2183-9.

32. Shin HD, Park BL, Kim LH, Lee HS, Kim TY, Bae SC. Common dnase i polymorphism associated with autoantibody production among systemic lupus erythematosus patients. Hum Mol Genet 2004;13:2343-50.

33. Yasutomo K, Horiuchi T, Kagami S, Tsukamoto H, Hashimura C, Urushihara M, et al. Mutation of dnase1 in people with systemic lupus erythematosus. Nat Genet 2001;28:313-4.

34. Napirei M, Karsunky H, Zevnik B, Stephan H, Mannherz HG, Moroy T. Features of systemic lupus erythematosus in dnase1-deficient mice. Nat Genet 2000;25:177-81.

35. Fenton K, Fismen S, Hedberg A, Seredkina N, Fenton C, Mortensen ES, et al. Anti-dsdna antibodies promote initiation, and acquired loss of renal dnase1 promotes progression of lupus nephritis in autoimmune (nzbxnzw)f1 mice. PLoS One 2009;4:e8474.

36. Seredkina N, Zykova SN, Rekvig OP. Progression of murine lupus nephritis is linked to acquired renal dnase1 deficiency and not to up-regulated apoptosis. Am J Pathol 2009;175:97-106.

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FIGURE LEGENDS

Figure 1. Circulating Neutrophil Extracellular Traps (NETs) Remnants.

(**A**) Serum NETs were determined using an ELISA measuring the DNA-MPO complex. Results are Relative Unit/ml given as median and interquartile range. The dotted line indicates the upper limit of normality (0.89). Here, it is reported serum NETs in all SLE, in all LN and in normal people. It is also, in parallel, shown NETs levels in LN patients split in the two subgroups divided according to the indications given in Materials and Methods (i.e. LN as onset, LN after 1year from the SLE onset).

(**B**) ROC curves showing specificity and sensitivity of the DNA-MPO assay for LN and SLE patients.

(**C**) Heat Map presenting correlations between circulating NETs levels and several laboratory parameters in the cohorts of patients with LN and SLE and in controls: in this analysis the color intensity is proportional to the Spearman's correlation coefficient that varies from a positive (red=+1) to an inverse (blue=-1) relationship where white corresponds to 0. Details on correlations are also reported in the two vertical columns, one indicating the numerical value of R2 while the second column indicates the P value in base Log 10. Where indicated, the P values were high statistically significant since the minimum for significance for Log10 P is 1.3 corresponding to 0.05.

(**D**) relationships between NETs remnants serum levels (x-axis) and major markers of lupus clinical activity, *i.e.* anti-dsDNA autoantibodies, C3 and C4. Only best fittings (R^2>0.5) are reported here.

Figure 2. Serum DNase activity.

(**A**) DNase activity was determined with a one-step assay based on fluorescence decrease of degrading Picogreen DNA dye/double-stranded DNA (dsDNA). In patients indicated with a square serum DNase activity was re-tested after treatment with Protein A/G. The triangle indicates a boy who presented a c.289_290delAC homozygous variant in *DNASE1L3.* Here, it is also in parallel

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reported DNase activity in LN patients split in the two subgroups divided according to the indications given in Materials and Methods (i.e. LN as onset, LN after 1year from the SLE onset).

(**B**) patients with LN, SLE and controls were subdivided according to their serum levels of NETs remnants (higher and lower than the normal level of 0.5 RU/ml). For all sub-groups, DNase activity is reported in the Y axis showing that low levels were present only in LN despite comparable circulating NETs in the other sub-groups. In the right panel it is shown that the 20% of LN patients with high circulating NETs (>0.5 MPO-DNA RU/ml) had low DNase vs 0 of the low circulating NETs sub-groups

(**C**) Sera with DNase activity in the lower range (that are indicated in squares in Figure2a) were pre-treated with Protein A (staphylococcus aureus) to remove potential inhibitors. In this case, 100 μl of Protein A (Sigma Aldrich, St Louis, MO) in PBS were incubated with 100 μl of serum for 2 hours. Unbound material was recovered after centrifugation (800 g x 30 min) and re-tested for DNase activity.

Figure 3. DNASE1 and DNASE1L3 levels.

(**A**) DNASE1 serum levels. A homemade ELISA assay has been utilized to test DNASE1. Results are expressed as ng/ml and represented as median and interquartile range.

(**B**) DNASE1L3 serum levels. For DNASE1L3 we utilized a commercial ELISA (LSBio kit, Seattle, USA). Results expressed as ng/ml are given as median and interquartile range. In this case, those LN patients who presented maximal variability in DNase activity were chosen for testing DNASE1L3 levels here including patients with low and patients with high DNase activity. For the broad distribution of values, ROC and normal limits were not calculated.

(**C**). Lack of correlation between serum levels of DNASE1 and DNASE1L3 in the cohorts of patients and controls who underwent the analysis of both enzymes.

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*The arrow indicates levels of DNASE1 and DNASE1L3 in the carrier of the c.289_290delAC homozygous variant in *DNASE1L3.*

Figure 4. NETs production and protein composition.

(**A)** *Ex vivo* NETs formation was evaluated as elastase and DNA release by resting neutrophils purified from patients with SLE (n=5), lupus nephritis (n=9) and from healthy controls (n=8). More cells (15 SLE, 18 LN and 27 controls) were utilized for stimulation with phorbol-12-myristate-13-acetate (PMA). Kinetics of NETs formation was analyzed in all supernatants utilizing the elastase method(13).

(**B**) In all experiments the release of elastase from stimulated neutrophils was highly correlated with the release of DNA.

(C) *Ex vivo* NETs production by isolated neutrophils purified from a SLE patients and stimulated with the homologous serum. Immunofluorescence for Histone 1 and 3 and staining for DNA are described in Supplemental Data. Arrows indicate the presence of extracellular material containing Histone1, Histone3 and DNA; actually, this material corresponds to NETs

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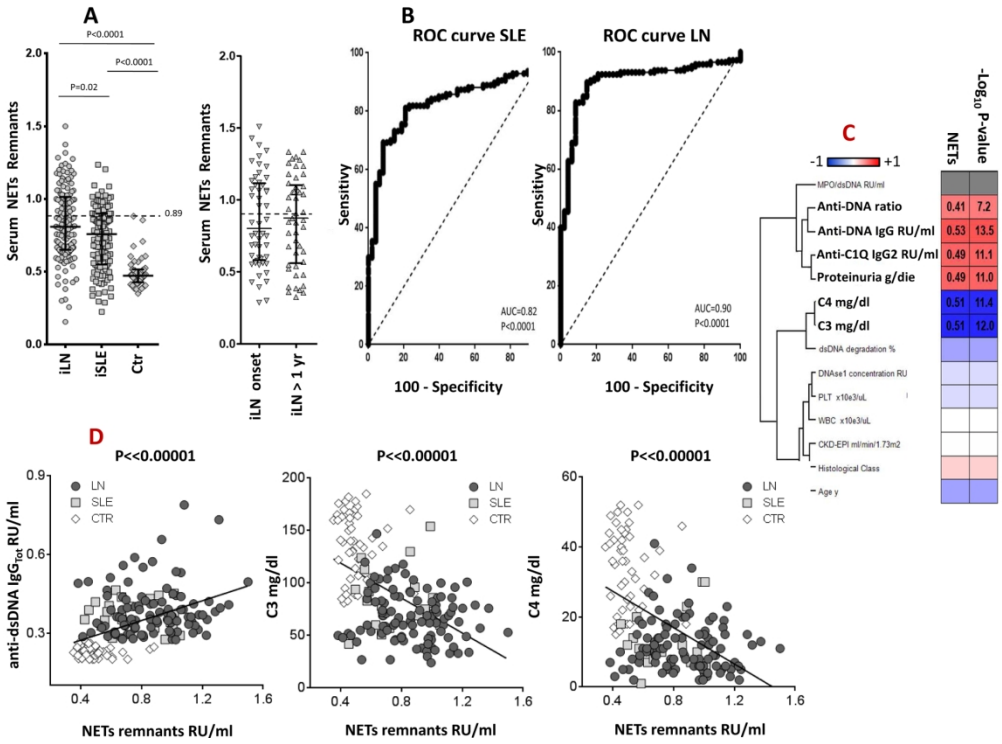

Figure 1

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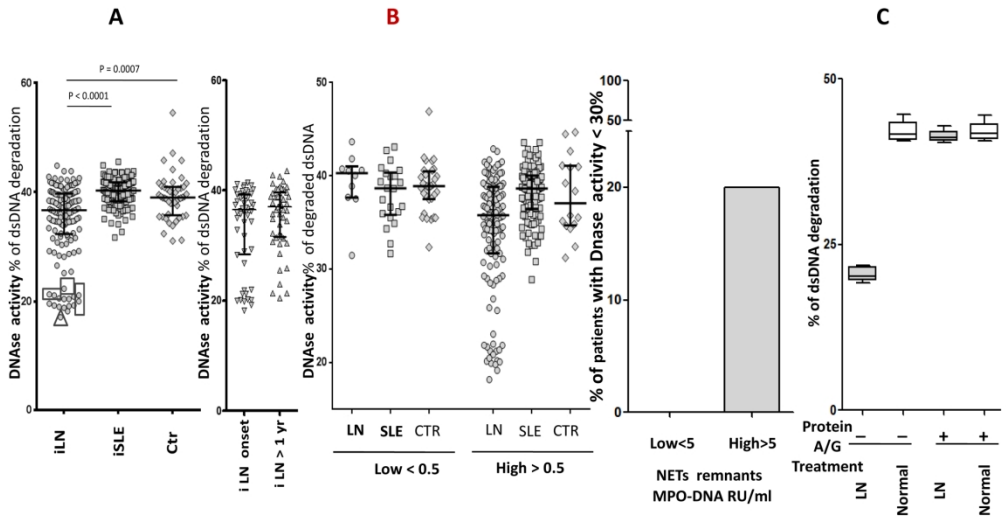

Figure 2

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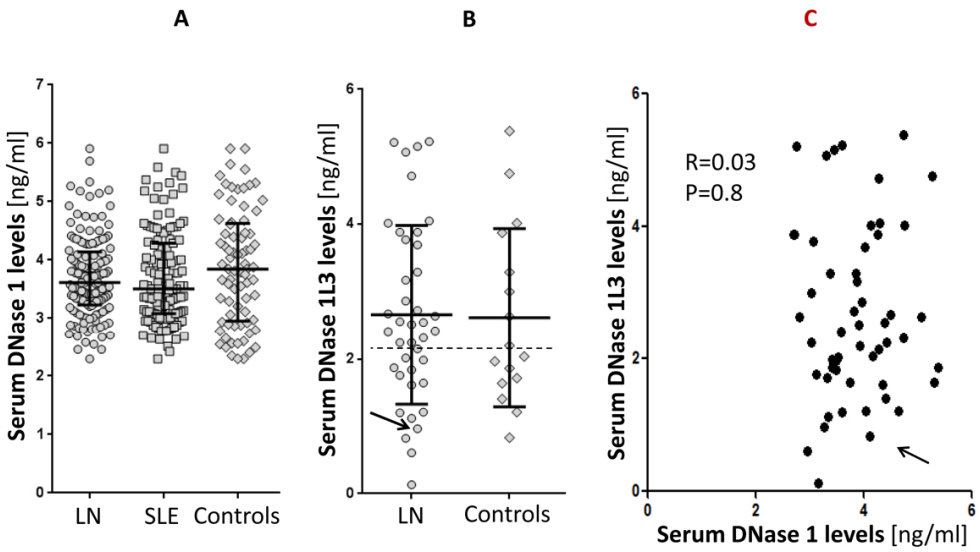

Figure 3

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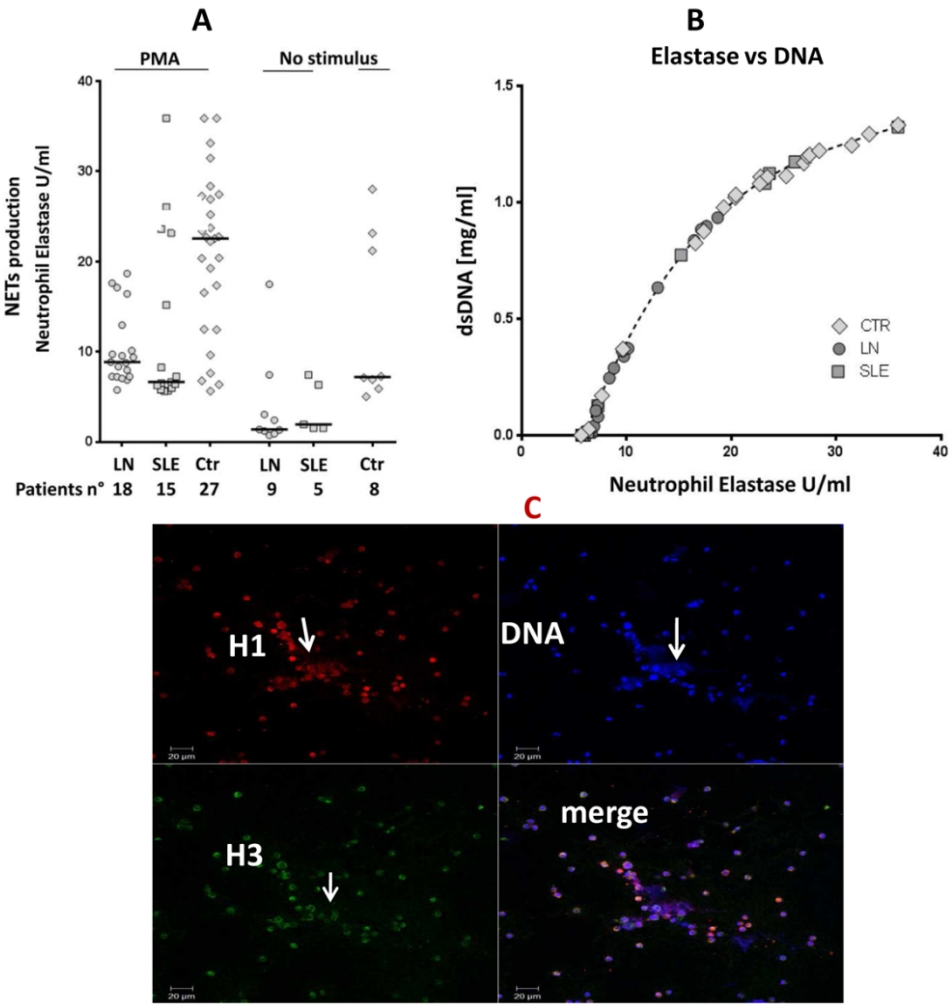

Figure 4

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Table 1A. Characteristics of iSLE, iLN, IgA, MN and controls.

Patients	iSLE	iLN	CTR	IgA	MN
Number	113	103	50	20	30
Female/Male (n)	100/13	90/13	43/7	16/4	6/24
Age (years)	35 * (22-51)	25* (8-43)	38* (19-50)	28* (15-30)	42* (35-48)
yrs from diagnosis of SLE	<0,3	-	-	-	-
yrs from diagnosis of LN	<0,3	<0,3			
Organ involvement: n(%)					
Kidney	0	103(110)			
Joint	49 (43)	42(41)			
Hematologic**	65(58)	83(80)			
Cutaneous (non rush)	13(12)	0			
Neurologic	3(3)	0			
SLEDAI	8+/-4	6+/-6			
C3 mg%	81+/- 42	91+/-30			
C4 mg%	13+/-12	14+/-12			
Proteinuria (g/24 hours)					
at diagnosis	0.01 (0.01-0.1)	2.41 (1.2-4.5)	NA	0.5	6.8
after 12 months	0.07 (0.02-0.1)	0.49 (0.2-1.1)	NA	0.4	2.4
Serum proteins (g/dl)					
at diagnosis	7.3 (6.9-7.7)	6.1 (5.3-6.8)	NA		
after 12 months	7.1 (6.6-7.7)	6.6 (6.3-7.08)	NA		
Therapy at diagnosis/after 12 months n(%)					
Steroids	82 (65)/26 (21)	78 (76)/62 (60)	NA/NA	12	0
Cytotoxic	0 (0)/0 (0)	25 (24)/0 (0)	NA/NA	-	20
Cyclosporine A	1 (1)/3 (2)	1 (1)/4 (4)	NA/NA	-	8
Azathioprine	5 (4)/0 (0)	6 (6)/21 (20)	NA/NA	-	
Mofetil Mycophenolate	3 (2)/0 (0)	7 (7)/14 (14)	NA/NA	-	
None/Hydroxychloroquine only	34 (31)/5 (4)	20 (19)/1 (1)	NA/NA	-	

Legend: NA= not available.

*data are given as median and interquartile ranges.

**For the presence of Hematologic changes, the SLEDAI 2K indication of WBC<3000 was considered.

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Table 1B. Renal and urinary characteristics in incident LN patients. Incident LN (iLN) was defined as the development of urinary symptoms of nephritis in patients with serum positivity for SLE markers and renal pathology typical of lupus nephritis. A sub-group of iLN presented the renal pathology as first symptom; a second sub-group presented the renal flare after 1 or more years from the SLE diagnosis.

Patients	**i LN** as onset	**i LN** after SLE
Number	53	50
Female/Male (n)	48/5	45/5
Age (years)	22 (8-40)	27 (18-43)
Pts <16yrs	3	0
yrs from LN	<0,3	<0,3
yrs from SLE	-	>1
Urinary abnormalities		
Casts	++	++
Proteinuria	2,5 (1,6-4,5)	2,1(1,3-4)
Red blood cells	+++	+++
Serum creatinine	0,7(0,5-1)	0,6(0,4-0,9)
Histological stage (%)		
I	0	0
II	7	10
III	25	28
IV	35	32
V	33	30
Therapy at diagnosis/after 12 months n(%)		
Steroids	40(76)/33 (60)	40(80)/10(20)
Cytotoxic	13(24)/0 (0)	15(30)/0(0)
Cyclosporine A	1 (1)/2 (4)	0(0)/0(0)
Azathioprine	3(6)/11(20)	0(0)/15(30)
Mofetil Mycophenolate	4 (7)/7 (14)	2(0,5)/10(20)
None/Hydroxychloroquine only	10 (19)/1 (1)	20(40)/5(10)

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